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Tilletia vankyi, a new species of reticulate-spored bunt fungus with non-conjugating basidiospores infecting species of *Festuca* and *Lolium*

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ABSTRACT

A bunt fungus, exhibiting a spore germination pattern unique to known reticulate-spored species of *Tilletia* was found infecting plants in seed production fields of *Festuca rubra* ssp. *rubra* (red fescue) and *F. rubra* ssp. *fallax* (Chewing's fescue) in Oregon, and in seed lots of *Lolium perenne* (perennial ryegrass) from Australia and Germany. Teliospores germinated to form 20–40 uninucleate, non-conjugating basidiospores, and colonies derived from single basidiospores produced teliospores in culture. In inoculation studies using single basidiospore colonies, perennial ryegrass and *L. perenne* ssp. *multiflorum* (Italian or annual ryegrass) were infected. A phylogenetic analysis, based on ITS region rDNA, eukaryotic translation elongation factor 1 alpha, and the second largest subunit of RNA polymerase II demonstrated that the fescue and ryegrass bunts are conspecific, and distinct from known species of *Tilletia*.

Introduction

While surveying *Lolium perenne* (ryegrass) seed samples from Australia in 1997, a single bunted seed filled with reticulately ornamented teliospores was found in each of two different samples of perennial ryegrass seed. Scientists at the Tianjin Entry-Exit Quarantine and Inspection Bureau in Tanggu, China, found a similar bunt fungus in a shipment of Australian perennial ryegrass seed in 2003, in German perennial ryegrass seed in 2005, and in *Festuca rubra* (red fescue) seed originating in the USA in 2003. Australian quarantine officials also intercepted a bunt in *F. rubra* from the USA in 2000. The two species of *Tilletia* with reticulately ornamented teliospores known to infect *Lolium* and *Festuca* are *T. contraversa*, the causal agent of dwarf bunt of wheat, and *T. lolii*, which only infects *Lolium* (Durán & Fischer 1961; Vánky 1994). However, the teliospore germination pattern of the bunts on *Lolium* and *Festuca* was different from that reported for both *T. contraversa* and *T. lolii*. *T. contraversa* is the subject of quarantine and considerable concern by both Australia and China, as well as a number

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of other countries. In 2005, 6.8 M kg of grass seed valued at $9 M was exported from the US to China (http://oregonstate.edu/international/CWG/3/WCY012806.pdf). The presence of *T. contraversa* or a new species of *Tilletia* in turf or forage grass seed produced in the US would have a serious impact on the US seed trade.

A multigene phylogenetic analysis of morphologically similar reticulate-spored *Tilletia* spp. on hosts in the subfamily *Pooideae* (*Poaceae*) was performed to determine whether the bunt fungi from *Festuca* and *Lolium* represent a single species and to determine how they are related to *T. contraversa* and *T. lolii*. A description of a new species of *Tilletia*, results of inoculation studies and surveys, and a comparison of morphological characters among ten closely related species of *Tilletia* are presented.

Materials and methods

Morphological characterization

Collections of *Tilletia* spp. examined as part of this study are listed in Table 1. Host names and authorities are listed as in the US Department of Agriculture PLANTS Database (http://plants.usda.gov). Voucher Material has been deposited in Herb. WSP (Washington State University, Pullman, WA – USA), Herb. BPI (Beltsville, MD – USA) and Herb. HUV (Tubingen, Germany).

Teliospores were mounted in Shear's mounting medium [50 ml 2 % (w/v) potassium acetate, 20 ml glycerol, 30 ml 95 % ethyl alcohol] and examined using DIC microscopy at ×1000. Teliospore diam (including exospore), thickness of exospore, and number of meshes per spore diam were recorded (Table 2). Teliospore shape, colour and exospore ornamentation, and sterile cell shape, colour and wall thickness were also recorded (Table 2).

Culturing

Grass seed samples were soaked 1–2 d in tap water to render the palea and lemma transparent, and examined at ×20 for detection of bunted seeds. For germination, teliospores were surface-sterilized in 0.26 % NaClO (5 % commercial bleach) in a 1.5 ml Eppendorf tube for 50 s, pelleted by centrifugation at approximately 13,000 ***g*** in a benchtop microcentrifuge for 10 s and rinsed twice with sterile, distilled water. Surface-sterilized teliospores were streaked on 1.5 % water agar (WA) and incubated at 5, 15 °C and room temperature (20–25 °C). Basidia and basidiospores were fixed and stained with Giemsa–HCl following Durán (1980).

Inoculation of plants

Inoculum of the putative new *Tilletia* species was derived from V21-711, one of the two bunted seeds found in seed samples of *Lolium perenne* from Australia. *T. contraversa* WSP 71413; (see Table 1) was also used in inoculation studies. After germination, single or polysporic basidiospore lines were grown on potato–sucrose agar (PSA) or M-19 agar (Trione 1964). Mycelium and sporidia (secondary basidiospores) for inoculation studies were grown in potato sucrose broth in 250 ml flasks on an orbital shaker at 250 rev min^{-1} at 15 °C. Surface-sterilized seeds of *L. perenne*, *L. perenne* ssp. *multiflorum*, and *Triticum aestivum* cv. 'Red Bob' were germinated on moistened filter paper in 9 cm diam Petri dishes or in moistened vermiculite in plastic storage boxes under laboratory conditions of temperature (18–25 °C) with light.

Plants were inoculated using either the coleoptile injection method, which is used for systemically infecting species of *Tilletia* (Fernandez & Durán 1978), or the boot inoculation method, which is used for nonsystemic species that infect through the developing florets (Carris *et al.* 2006). For the coleoptile inoculation method, a suspension of sporidia and mycelium (*ca* 20,000 sporidia ml^{-1}) was injected into the coleoptile at the seedling stage using a hypodermic needle (30 G needle for *Lolium* spp., 22 G needle for *Triticum aestivum*). For boot stage inoculation, a suspension of sporidia and mycelium (*ca* 20,000 sporidia ml^{-1}) was injected into plants at the boot stage, just prior to the emergence of the flag leaf. Polysporic inoculum derived from multiple teliospores was used for boot inoculation of 25 plants each of *L. perenne*, *L. perenne* ssp. *multiflorum*, and *T. aestivum*. Polysporic inoculum of V21-711 was also used for coleoptile inoculation of 25 plants each of *L. perenne* ssp. *multiflorum* and *T. aestivum*. Inoculum derived from a single basidiospore was used for boot inoculation of 25 plants of *L. perenne* ssp. *multiflorum*. Polysporic inoculum of *T. contraversa* (WSP 71413) was used for coleoptile and boot inoculation of 25 plants each of *T. aestivum* and *L. perenne* ssp. *multiflorum*.

Plants inoculated at the coleoptile stage were incubated in covered plastic storage boxes for one month at 6 °C in the dark and then transferred to the greenhouse. Seedlings were planted five per 15 cm diam pot in a pasteurized potting mix and grown in a greenhouse at 20–25 °C with 16 h light, supplemented with artificial light when necessary. Perennial ryegrass plants were overwintered in an outside courtyard between greenhouses to induce heading. Plants inoculated at the boot stage were placed in a mist chamber for 3 d after inoculation prior to being moved to the greenhouse.

Nucleic acid extraction and PCR amplification

Mycelium for DNA extraction was grown in shaker flasks at 125 rev min^{-1} in 100 ml liquid potato–dextrose broth at room temperature or 15 °C under ambient light conditions. Mycelium was harvested by centrifugation and freeze-dried. Alternatively, DNA was extracted directly from actively growing surface mycelium scraped from PSA or M-19 plates. DNA was extracted with the PureGene DNA extraction kit (Gentra Systems, Madison, WI) according to the manufacturer's instructions using approximately 15 mg dried tissue or 50 mg fresh mycelium. For specimens with teliospores that did not germinate, one sorus was crushed in a 1.5 ml microcentrifuge to release the teliospores, which were then lysed with the buffer provided in the PureGene kit and extracted according to the manufacturer's instructions.

Individual genes were amplified in a 50 µl reaction on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) or I-Cycler (Bio-Rad, Hercules, CA) using the following primers: either EF1-526F (5′-GTCGTYGTYATYGGHCAYGT-3′) as the forward primers and EF1-1567R (5′-ACHGTRCCRAT

Table 1 – Isolates used in this study

Taxon	Voucher/Culture numbers[a]	Year	Host	Origin	Collector/ source	GenBank accession numbers[b]
Tilletia brevifaciens.	HUV 20.802 (CBS 121948)	2004	*Thinopyrum intermedium* (Host) × *Elymus repens*	Poland	*K. Vánky*	
	WSP 68945 (Vánky 412)	1999	*T. intermedium*	Austria	*K. Vánky*	
T. bromi	WSP 71271 (LMC 148)	1992	*Bromus tectorum*	US (WA)	*L. Carris*	
	WSP 71272 (LMC 167)	1992	*Bromus arvensis*	US (WY)	*L. Carris*	
	WSP 71273 (LMC 75)	1991	*Bromus hordeaceus* ssp. *hordeaceus*	US (WY)	*L. Carris*	
	WSP 71315	2003	*Bromus* sp.	US (Chinese intercept)	*G. Huang*	
T. caries	WSP 71304 (DAR 73302)	1997	*Triticum aestivum*	Australia	*G. Murray*	
	WSP 71303 (LMC J-19; CBS 121951)	1995	*T. aestivum*	Sweden	*L. Carris*	
T. contraversa	WSP 71280 (LMC 282; CBS 121952)	1994	*T. aestivum*	US (OR)	*L. Carris*	
	WSP 71413 (LMC 177)	1985	*T. aestivum*	US (UT)	*B. Goates*	
	WSP 71301 (LMC 94)	1991	*Bromus marginatus*	US (ID)	*L. Carris*	
	WSP 69062 (Vánky 528)	1984	*T. aestivum*	Germany	*K. Vánky*	
T. elymi	WSP 71274 (LMC 158)	1992	*Elymus glaucus* ssp. *glaucus*	US (WY)	*L. Carris*	
T. fusca	WSP 71275 (LMC 141)	1971	*Vulpia microstachys* var. *microstachys*	US (WA)	*L. Carris*	
T. goloskokovii	WSP 69688 (LMC 321)	1995	*Apera interrupta*	US (WA)	*L. Carris*	
	WSP 69687 (LMC 315-95-3)	1995	*A. interrupta*	US (WA)	*L. Carris*	
	WSP 71281 (LMC 238-2)	1993	*A. interrupta*	US (WA)	*L. Carris*	
T. laevis	WSP 71278 (LMC178; CBS 121949)	1971	*T. aestivum*	US	*L. Carris*	
	WSP 71300 (Vánky 766; CBS 121950)	1988	*T. aestivum*	Iran	*K. Vánky*	
	WSP 71302 (LMC 98-194)	1998	*T. aestivum*	Australia	*L. Carris*	
T. laguri	HUV 16.352	1992	*Lagurus ovatus*	Italy	*K. Vánky*	
T. lolii	WSP 71298 (Vánky 767)	1990	*Lolium rigidum*	Iran	*K. Vánky*	
T. lolioli	WSP 71305 (Vánky 763)	1990	*Loliolum subulatum*	Iran	*K. Vánky*	
T. secalis	WSP 71279 (LMC 255)	1993	*Secale cereale* L.	US (ID)	*L. Carris*	
T. sphaerococca.	WSP 71314	2003	*Agrostis stolonifera*	US (Chinese Intercept)	*G. Huang*	
T. togwateei	WSP 71276 (LMC 153)	1992	*Poa reflexa*	US (WY)	*L. Carris*	
	WSP 71277 (LMC 169)	1992	*P. reflexa*	US (WY)	*L. Carris*	
T. trabutii	WSP 71299 (Vánky 764)	1990	*Hordeum murinum* ssp. *glaucum*	Iran	*K. Vánky*	
	VPRI 32106	2005	*H. murinum* ssp. *leporinum*	Australia	*I. Pascoe*	
T. vankyi	WSP 71266 (V21-713; CBS 121953)	1997	*Lolium perenne*	Australia	*L. Carris*	
	WSP 71263 (V21-711; CBS 121954)	1997	*L. perenne*	Australia	*L. Carris*	
	WSP 71270 (FF1-1)	2005	*F. rubra* ssp. *fallax*	US (OR)	*S. Alderman*	
	WSP 71316	2003	*Festuca rubra* ssp. *fallax*	US (Chinese Intercept)	*G. Huang*	
	no voucher available	2002	*L. perenne*	Australia (Chinese Intercept)	*G. Huang*	
	no voucher available (FF7/8)	2005	*F. rubra* ssp. *fallax*	US (OR)	*S. Alderman*	
	WSP 71268	1951	*F. rubra*	US (OR)	*J. Hardison*	Not sequenced
	WSP 71267 (BRIP 27634)	2000	*Festuca* sp.	US (Australian intercept)	*R. Eichner, R. Shivas*	Not sequenced
	LJF 2005 GS-18-20	2005	*L. perenne*	Germany (Chinese intercept)	*G. Huang*	Not sequenced

a CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; HUV, Herbario Ustilaginales Vánky, Tübingen, Germany; Vánky, *Ustilaginales Exsiccata* by K. Vánky; WSP, Washington State Plant Pathology Herbarium, Pullman, WA, USA.
b Sequences of EF1A, ITS, and RPB2 gene regions, were deposited in GenBank as accessions EU257524-EU257620.

ACCACCRATCTT-3′) as the reverse primer; ITS5 (5′-GGAA GTAAA AGTCGTAACAAGG-3′) or ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) as forward primers and ITS4 (5′-TCCTCCGCT TATTGATATGC-3′) as the reverse primer; RPB2-740F (5′-GAT GGACGCGGTTTGTAATG-3′) and RPB2-1365R (5′-TCGAAGAG CYAACACTGAGACG-3′) (White *et al.* 1990; *Primers for elongation factor 1-α (EF1-α)*; http://ocid.nacse.org/research/deephyphae/EF1primer.pdf, Lynne Carpenter-Boggs, pers. comm.). Forward primer EF1-636F (5′-TCAACGTCGTYGTYATCGG-3′) was designed during this study for isolates in which the EF1-526F

Table 2 – Infection type, morphology, and germination of *Tilletia* species compared in study

Taxon	Infection type	Teliospore morphology				Sterile cells		Teliospore germination[a]		Primary basidiospores[a]				
		Colour	Diam (μm)	Exospore depth (μm)	Meshes per spore diam	Diam (μm)	Wall thickness (μm)	Temp.	Days	Shape	Number	Size (μm)	Nuclei per basidiospore	Conjugation
Tilletia brevifaciens	Systemic	Pale to medium yellowish-brown	17–21	1.8	4–6	7–14	0.5–1	5–15C	>21	Filiform	6–8	81–88 × 2.5	1	Yes
T. bromi	Systemic	Pale to medium reddish brown	18–29	1–3	5–10	14–26	1–3	5C	>14	Filiform	8–28	70–88 × 2–2.5	1	Yes
T. contraversa	Systemic	Pale to medium reddish brown	15–27	1.8–3.5	5–7	7–22	1–1.3	5C	>21	Filiform	14–30	57–84 × 2–2.5	1	Yes
T. goloskokovii	Systemic	Pale to dark fuscous brown	18–30	1–3.2	4–8	15–28	1–2.5	5–10C	>24	Filiform	22–24	48–66 × 2.5	1	Yes
T. laguri	Systemic	Pale to medium reddish-brown	18–20	1.8	6–8	10–15	1–1.8	N/a	N/a	N/a	N/a	N/a	N/a	N/a
T. lolii	Systemic	Pale yellow to yellow-brown	16–27	1–2.5	5–10	9–19	1–2.5	5–15C	3–10	Fusiform	4–12	20–30 × 2.5–4	1	Yes
T. lolioli	Systemic	Yellow- to reddish-brown	20–27	1–1.5	6–10	10–18	0.5–1	5–15C	7–21	Filiform	9–21	56–80 × 2.5	1	Yes
T. sphaerococca	Systemic	Pale yellow-brown to dark brown	21–30	1.5–3.5	5–10	10–23	1–2.5	5C	22–30	Filiform	26–32	66–76 × 2–2.5	1	Yes
T. trabutii	Systemic	Pale to medium reddish-brown	20–23	1.3–1.8	4–8	10–16	1–1.8	5–15C	>14	Filiform	12–15	66–75 × 2.5	1	Yes
T. vankyi	Local	Pale to medium reddish-brown	18–30	1–2	5–8	10–21	0.5–1.8	5–15C	5–14	Filiform	24–40	53–74 × 1.5–3.5	1	No

a N/a = no data available.

and EF1-1567R primer combination did not produce an amplification product.

Standard cycling parameters with a 55 °C annealing temperature were used for the EF1A, RPB2, and ITS gene regions. For isolates that were difficult to amplify using the EF1A primers the following parameters were used: (1) 10 min at 95 °C; (2) 35 s at 94 °C, 55 s at 66 °C, 1 min 30 s at 72 °C for nine cycles decreasing by 1 °C each cycle; (3) 35 s at 94 °C, 55 s at 56 °C, 1 min 30 s at 72 °C for 35 cycles; (4) final extension 10 min 72 °C. PCR products were purified using ExoSAP-IT (USB, Cleveland, OH) according to the manufacturer's instructions. Amplified products were sequenced with the BigDye version 3.1 dye terminator kit (Applied Biosystems, Foster City, CA) on an ABI 3100 automated DNA sequencer. The respective PCR primers were also used as sequencing primers for all the genes.

Sequence analysis

Raw sequences were edited using Sequencher version 4.5 for Windows (Gene Codes Corporation, Ann Arbor, MI). Alignments were edited with GeneDoc 2.6.03 (http://www.psc.edu/biomed/genedoc/). The three genes were aligned individually and then concatenated into a single alignment. Each gene was analysed separately through the use of data partitions and a combined three-gene analysis was performed for all available taxa. For one isolate of *Tilletia togwateei* and the sole isolate of *T. laguri* the EF1A could not be sequenced. These two isolates were included in the combined analysis with missing data for this gene region.

Trees were inferred by MP using the heuristic search option with the random addition sequence (1K replications) and the branch swapping (tree bisection–reconnection) option of PAUP version 4.0b10 (Swofford 2002). For MP analyses, a limit of ten trees per random addition sequence was enforced with a MAXTREE limit of 10K. All aligned positions were included. All characters were unordered and given equal weight during the analysis. Gaps were treated as missing data and trees were midpoint rooted. Relative support for branches was estimated with 1K BS replications (Felsenstein 1985) with MULTREES off and ten random sequence additions per BS replicate for the MP BS analysis. A NJ BS analysis (1K replications) was also performed using the model determined by Modeltest 3.7 (Posada & Crandall 1998) to be the best fit for the three genes combined.

The partition homogeneity test with 1K replicates as implemented in PAUP (Swofford 2002) was used to determine if significant incongruence among genes existed. In addition, a reciprocal 70 % NJ BS criterion as in Reeb *et al.* (2004) was used to assess topological incongruence among the genes (Mason-Gamer & Kellogg 1996). BS values were generated for each individual gene, each combination of two of the three genes and the three genes combined using an NJ BS of 1K replicates with distance settings as determined by Modeltest 3.7 (Posada & Crandall 1998) for each dataset.

Surveys

A survey of *Tilletia* spp. associated with perennial ryegrass in Australia was conducted by L. M. C. and L. A. C. in cooperation with Gordon Murray, New South Wales Agriculture, Wagga Wagga Agricultural Institute, from 21 Aug.–9 Sep. 1999. As part of the survey, 147 seed samples collected during 1996–1998 were examined using a modification of the seed wash protocol used for the US National Karnal Bunt Survey (Peterson *et al.* 2000). Thirty-nine samples were from commercial perennial ryegrass seed production fields in New South Wales Kangaroo Valley, Riverina and Wrightson regions, and 108 samples were from perennial ryegrass growing as weeds in other crops that were collected by scientists at Charles Sturt University and New South Wales Agriculture as part of an herbicide resistance study. The weed samples were collected from New South Wales (59 samples) and Victoria (40 samples), South Australia (three samples), Western Australia (three samples) and the remainder from unspecified locations in Australia.

Twenty-five grams of seed in 100 ml tap water with two drops of Tween-20 were placed in a 500 ml flask on a rotary shaker at 200 rev min^{-1} for 10 min. The seed suspension was passed through a 53 μm-pore sieve into a clean 600 ml beaker. The suspension from the beaker was poured through a 25 μm-pore sieve, and spores and debris remaining on the sieve were washed into a 15 ml conical centrifuge tube and centrifuged for 3 min at 200 rev min^{-1}. The supernatant was discarded and the pellet was resuspended in 100 μl Shear's mounting medium. The suspension was transferred to two to four microscope slides, a 22 × 50 mm coverslip was placed on each slide, and the slide was sealed with fingernail varnish. The slides were examined at ×125 using a compound microscope. Seed samples with >100 teliospores/25 g seed were soaked overnight in tap water to render the palea and lemma transparent and examined under a dissecting microscope to find individual bunted seeds.

During 2005, a survey of fine fescue fields was initiated in Oregon by S. C. A. During the first week of July 2005, 50 seed heads were collected arbitrarily along each of four transects (200 seed heads total) arranged in a diamond pattern from each of 21 fine fescue fields in Marion Co., OR, including 11 fields of *Festuca rubra* ssp. *fallax* (Chewing's fescue) and ten fields of *F. rubra* ssp. *rubra* (creeping red fescue). Seed heads from each transect were placed in paper bags (50 seed heads per bag) and stored at room temperature until processed. Each seed head was gently threshed by hand, and the seeds from each individual seed head were examined under a dissecting microscope for the presence of partially or fully bunted seeds. All seed heads were examined within ten weeks of collection. The number of seed heads with bunted seed and the number of bunted seeds per head were recorded. Teliospores from bunted seeds were surface-sterilized and plated on WA for germination as previously described.

Results

Morphology

The morphological and germination characters for ten *Tilletia* spp. included in this study are summarized in Table 2. Morphological characters associated with teliospores and sterile cells overlap among all species (Figs 1F–H, 2A–H, 3A–H). The size, shape, and number of primary basidiospores were similar among most of the species studied, except *T. lolii*, which

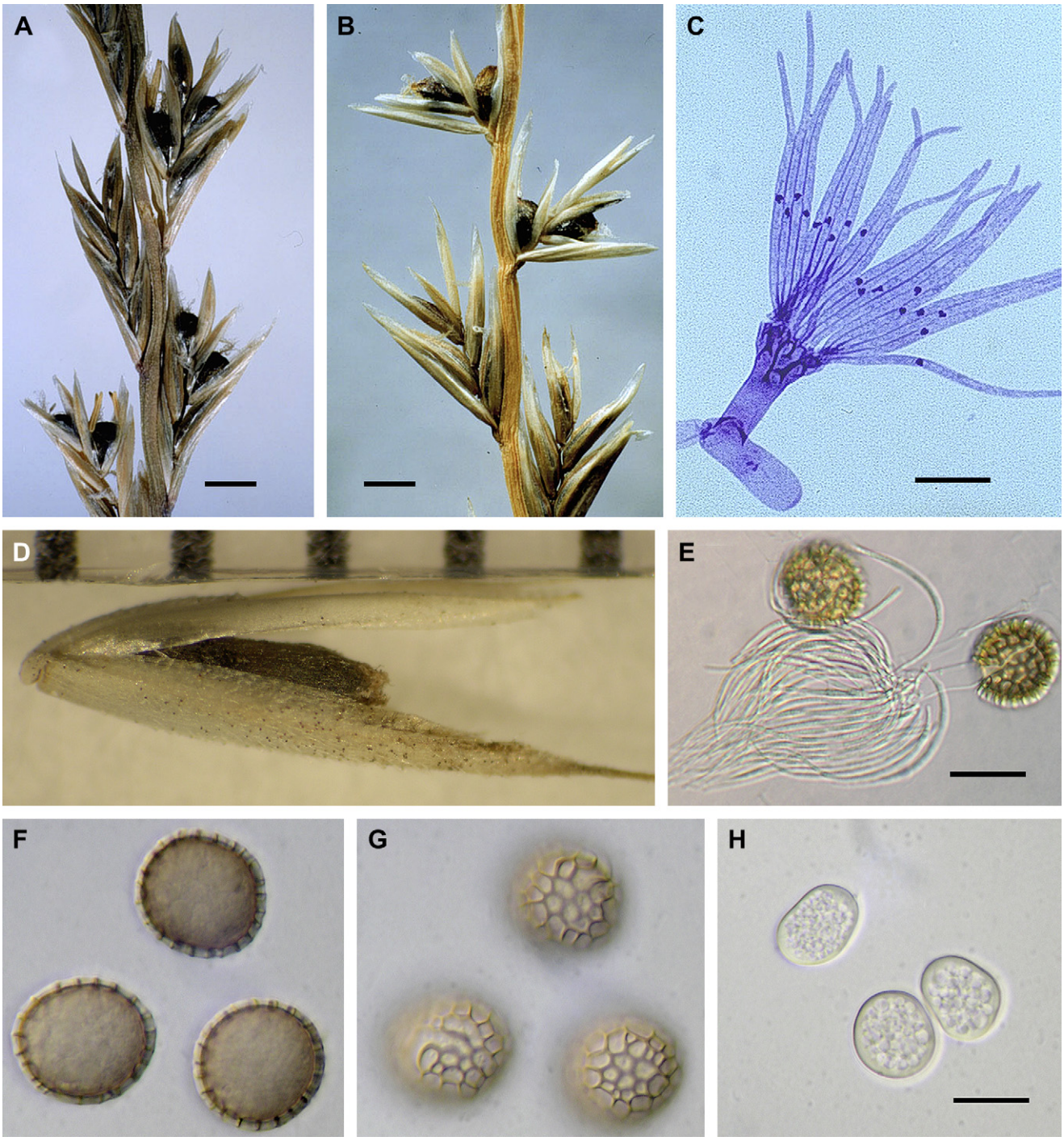

Fig 1 – *Tilletia vankyi*. (A) Artificial inoculation on *Lolium perenne* ssp. *perenne* using strain V21-711 from Australia (CBS 121954). Bar = 5 mm. (B) Artificial inoculation on *L. perenne* ssp. *multiflorum* using strain V21-711 from Australia CBS 121954. Bar = 5 mm. (C) Whorl of basidiospores stained with Giemsa–HCl to show nuclear condition (strain V21-711). Bar = 20 μm. (D) Bunted seed of *Festuca rubra*. Units = 1 mm. (E) Teliospore, basidium, and basidiospores from *Festuca rubra* (WSP 71270). Bar = 25 μm. (F–H) Teliospores and sterile cells, V21-711 (WSP 71263). Bar = 15 μm. (F) Teliospores, plane view. (G) Teliospores, surface view showing reticulate ornamentation. (H) Sterile cells.

produced relatively few, fusiform spores per basidium. Primary basidiospores of the nine species that we were able to germinate were uninucleate, based on Giemsa–HCl staining, with adjacent, compatible basidiospores conjugating rapidly after formation in all species except *T. vankyi* (Table 2). Teliospores from the two available specimens of *T. laguri*, including the holotype, did not germinate, and germination data were not provided in the only publication on this species (Zhang *et al.* 1995). Six of 20 single-basidiospore-derived colonies of V21-711, and three of 13 single-basidiospore-derived

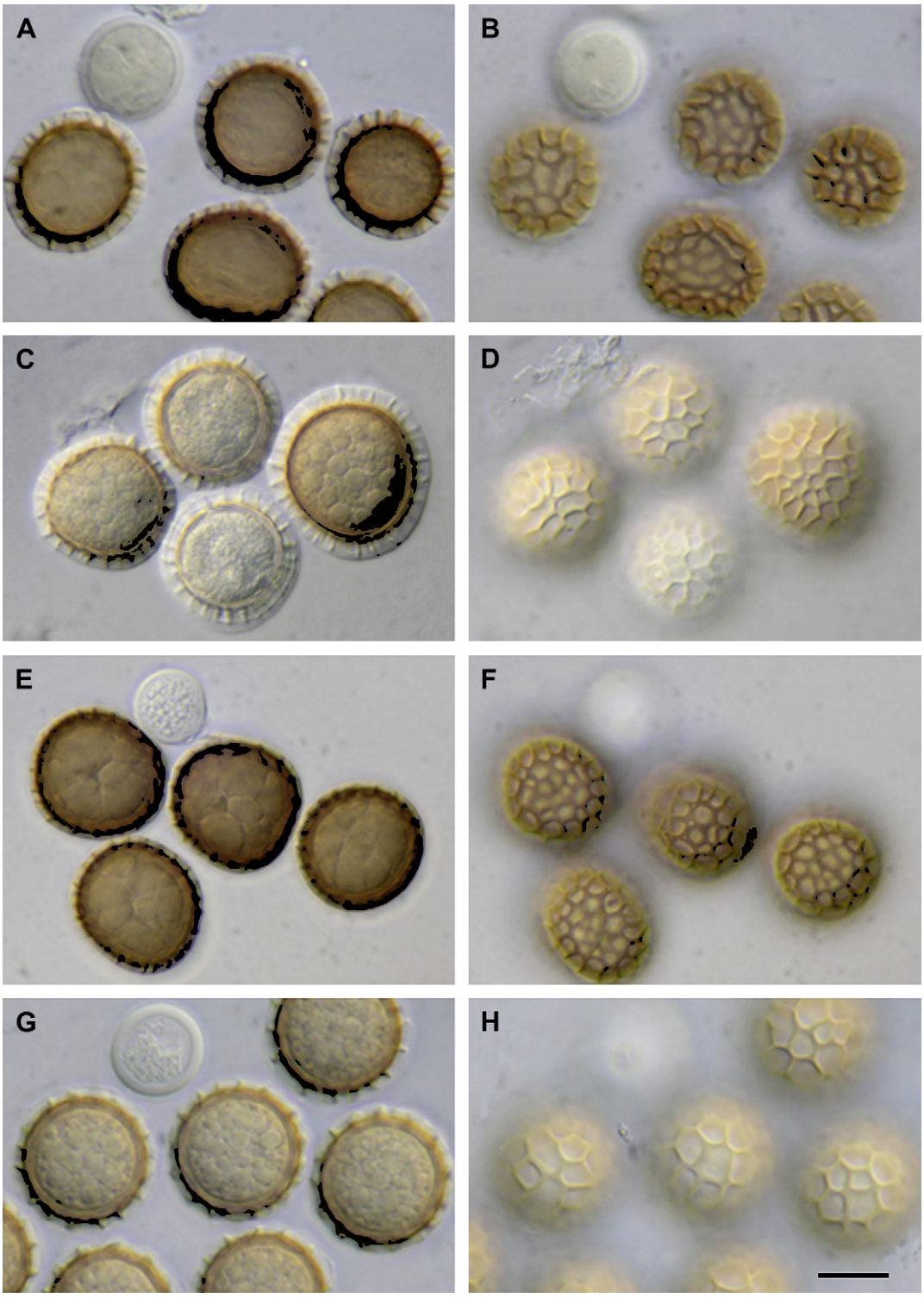

Fig 2 – *Tilletia* spp. (A–B) *T. laguri* (isotype HUV 16352). (C–D) *T. lolii* (WSP 71298). (E–F) *T. lolioli* (WSP 71305). (G–H) *T. trabutii* (WSP 71299). (A, C, E, G) Teliospores, plane view. (B, D, F, H) Teliospores, surface view showing reticulate ornamentation. Bar = 10 μm.

colonies of V21-713 produced teliospores in culture when grown on PSA slants at 15 °C in the dark. Teliospores produced in these cultures germinated in a manner indistinguishable from those formed *in planta*.

Greenhouse inoculations

Polysporic lines of *Tilletia* sp. V21-711 injected into *Lolium perenne* and *L. perenne* ssp. *multiflorum* at the boot stage resulted in

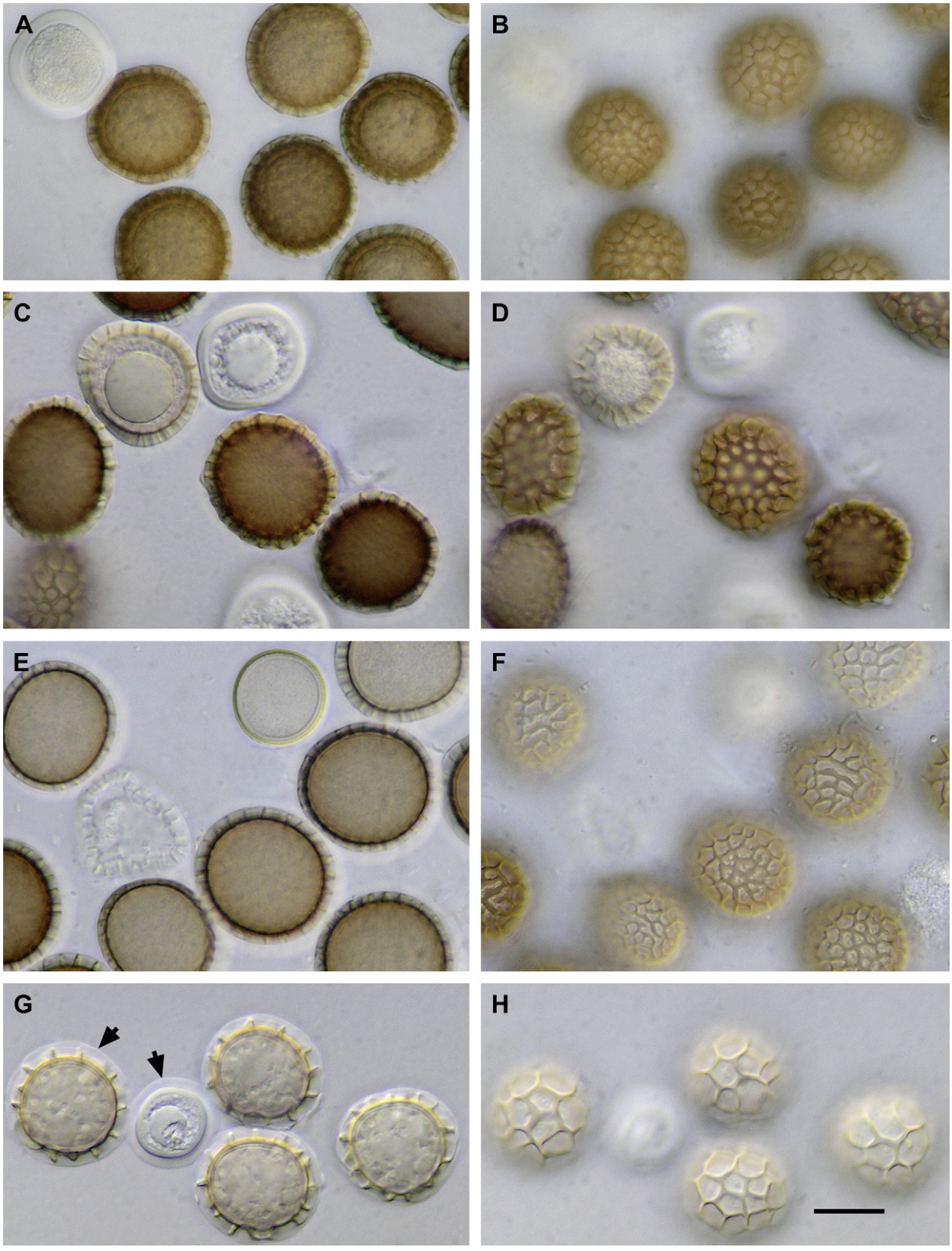

Fig 3 – *Tilletia* spp. (A–B) *T. bromi* (WSP 71271). (C–D) *T. goloskokovii* (WSP 69688). (E–F) *T. sphaerococca* (Chinese intercept). (G–H) *T. brevifaciens* (WSP 68945). (A–C) (E, G). Teliospores, plane view. (B, D, F, H) Teliospores, surface view showing reticulate ornamentation. Bar = 10 μm.

infection of 25 and 29 % of inoculated plants, respectively. Only a few seeds per head were bunted on infected plants. Polysporic lines of *Tilletia* sp. V21-711 injected into the coleoptiles of annual ryegrass seedlings resulted in infection of 2 % of the inoculated plants, and infected plants were dwarfed to approximately half the height of control plants. Inoculation of annual ryegrass plants at the boot stage with inoculum derived from single basidiospores resulted in infection in 33 % of the heads that developed.

Inoculation of wheat plants at the boot stage with polysporic inoculum of *Tilletia* sp. V21-711 resulted in infection in only one plant with one partially bunted seed formed on the inoculated seed head. In contrast, when polysporic *T. contraversa* inoculum was used to inoculate wheat, the coleoptile method resulted in 70 % infection, and the boot inoculation resulted in 27 % infection. When polysporic *T. contraversa* inoculum was used to inoculate annual ryegrass plants at the coleoptile stage, 16 % of the plants were infected. Boot inoculation resulted in 25 % infected plants.

Ryegrass and fescue surveys

Reticulate teliospores (22–30 μm diam) were present in 19 % of the Australian *Lolium perenne* seed samples, with a higher percent of spores in commercial field samples (51 %) than from weed samples (7 %). Tuberculate teliospores (28–35 μm diam) morphologically similar to those of *Tilletia walkeri* were found in 18 % of the seed samples, also with a higher percentage of spores in samples from commercial ryegrass fields (39 %) than from weed samples (11 %). Most of the 25 g seed washes contained fewer than 30 teliospores of either reticulate or tuberculate type. Four samples contained >100 tuberculate teliospores per 25 g seed and two samples contained >100 reticulate teliospores per 25 g sample. Two bunted seeds, one filled with tuberculate teliospores and one with reticulate teliospores, were found in these seed samples. Teliospores from the bunted seeds failed to germinate using the methods previously described.

During the Oregon fine fescue survey, one seed head with a single fully bunted seed was found in each of two Chewings fescue fields. In a third Chewings fescue field a seed head with 39 fully bunted seeds was found (the remaining 31 seeds on the head appeared healthy), with six additional bunted seeds found among seed shattered from heads prior to examination. All infected heads included both bunted and healthy seeds. Bunted seeds were not detected in the remaining fields. Teliospores from bunted fine fescue seeds germinated as previously described.

Phylogenetic analysis

Excluding primer binding site regions and 5′ and 3′ regions of the genes with excessive amounts of missing data, the combined alignment consisted of EF1A (721 bp), ITS (649 bp), and RPB2 (554 bp) sequences for 33 isolates of *Tilletia* spp. infecting grass hosts in the *Pooideae* for a total of 1924 characters. Of these, 164 were parsimony-informative, 1691 were constant and 69 were variable but not parsimony-informative. Trees were midpoint rooted. Modeltest 3.7 determined the following settings as the models best fitting the individual genes and all possible combinations of genes: (1) EF1A – TrN + G Base = (0.2343 0.3156 0.2554) Nst = 6 Rmat = (1.0000 1.5664 1.0000 1.0000 8.6007) Rates = gamma Shape = 0.1360 Pinvar = 0; (2) ITS – HKY + I + G Base = (0.2480 0.2288 0.2118) Nst = 2 TRatio = 1.6461 Rates = gamma Shape = 0.0075 Pinvar = 0.6427; (3) RPB2 – HKY + G Base = (0.1661 0.2805 0.3180) Nst = 2 TRatio = 2.1926 Rates = gamma Shape = 0.1826 Pinvar = 0; (4) EF1A and ITS – TrN + I + G Base = (0.2393 0.2730 0.2363) Nst = 6 Rmat = (1.0000 1.9153 1.0000 1.0000 7.4236) Rates = gamma Shape = 0.7926 Pinvar = 0.7379; (5) EF1A and RPB2 – TrN + I + G Base = (0.2054 0.3002 0.2861) Nst = 6 Rmat = (1.0000 3.1632 1.0000 1.0000 7.7506) Rates = gamma Shape = 0.6545 Pinvar = 0.5790; (6) ITS and RPB2 – Base = (0.2059 0.2530 0.2640) Nst = 2 TRatio = 2.1364 Rates = gamma Shape = 0.5406 Pinvar = 0.6584; (7) EF1A, ITS and RPB2 – TrN + I + G Base = (0.2185 0.2761 0.2618) Nst = 6 Rmat = (1.0000 3.1516 1.0000 1.0000 6.5386) Rates = gamma Shape = 0.6889 Pinvar = 0.6943.

The partition homogeneity test as implemented in PAUP indicated significant conflict among all possible combinations of the data partitions ($P = 0.01$ for all). Results of the 70 % reciprocal NJ BS showed incongruence between trees (not shown) in the placement of *T. brevifaciens*, the species on *Thinopyrum intermedium* (intermediate wheatgrass) and the isolate from *Secale cereale*, which we have identified as *Tilletia secalis*. The RPB2 data placed *T. brevifaciens* in a clade with the wheat bunts (78 %) and placed *T. secalis* with *T. trabutii* (99 %). The EF1A data placed *T. brevifaciens* outside the wheat bunt clade and placed *T. secalis* with *T. brevifaciens* (100 %). The ITS gene tree did not support any of the species clades. The clade containing *T. vankyi* was present in both EF1A and RPB2 gene trees (70 and 100 %, respectively). The EF1A/RPB2 NJ analysis and the EF1A/ITS NJ analysis both identified all species-level clades found in the three-gene combined MP tree although the support for *T. vankyi* was lower (62 and 54 % respectively). The ITS/RPB2 analysis placed *T. brevifaciens* inside the wheat bunt clade (76 %) and *T. secalis* with *T. trabutii* (98 %).

MP phylogenetic analysis of the combined alignment resulted in 196 equally parsimonious trees (length = 360, CI = 0.703, RI = 0.847, RC = 0.595, HI = 0.297) differing in the arrangement of the interior branches (trees not shown). Species-level clades were present in all trees. Fig 4 shows one randomly chosen MP tree generated for the combined alignment, with MP BS values above the branches. Only BS values 70 % or greater are shown. Thickened branches indicate the branch was supported in the strict consensus tree. The combined three-gene NJ analysis generally agreed with the MP analysis (tree not shown) and NJ BS values are shown underneath the branches on the MP tree for comparison.

Combined analysis of all three genes identified a clade containing five isolates of *T. vankyi* on *Festuca rubra* and *Lolium perenne* (100 % MP and 90 % NJ BS support). *Tilletia vankyi* was distinct from but grouped with two other species, *T. laguri* from *Lagurus ovatus* and *T. lolii* on *Lolium rigidum* (91 % MP). Other species distinguished include *T. goloskokovii* (100 % MP and NJ), *T. bromi* (100 % MP and NJ), *T. trabutii* (100 % MP and NJ), and *T. brevifaciens* (100 % MP and NJ). A clade containing *T. caries*, *T. contraversa*, and *T. laevis* was supported at the 87 % MP and 94 % NJ BS level, but the three species could not be distinguished from one another with any level of support.

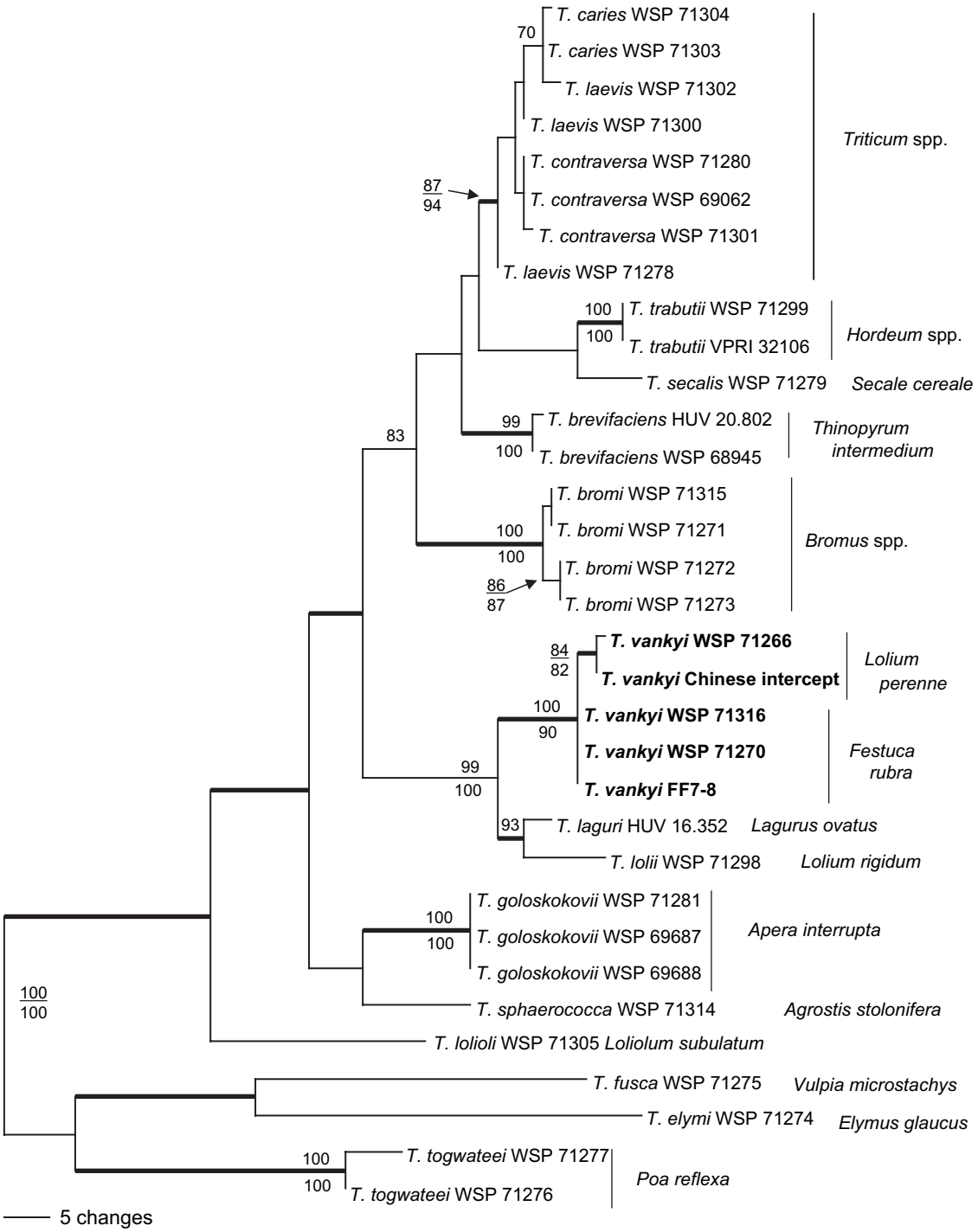

Fig 4 – Midpoint rooted phylogram showing one of 196 equally parsimonious trees resulting from a heuristic search of combined dataset consisting of EF1A, ITS and RPB2 gene regions (see text for details). Branches appearing in the strict consensus tree are indicated by thickened lines. Confidence levels are indicated by MP BS values above the branches and NJ BS values below the branches with only values greater than or equal to 70 % shown.

The isolates of *T. vankyi* from *Lolium* differed from the *Festuca* isolates by 2 C/T substitutions in the EF1A and the WSP 71266 isolate differed from all others by one C/T substitution at one position in the ITS. All other alignment positions were identical for a sequence identity of 1888/1891 positions (99.8 %). In comparison, isolates of *T. bromi* had 1901/1904 (99.8 %) and *T. goloskokovii* 1829/1829 (100 %) identical positions. Isolates in the wheat bunt complex showed one substitution in EF1A, four substitutions (three of which were ambiguous in several isolates) and two insertion/deletion (gapped positions) in the ITS, and three substitutions in RPB2 for a total of 1796/1806. Only one substitution in the RPB2

distinguished the three isolates of *T. contraversa* from *T. laevis* and *T. caries*.

Taxonomy

Tilletia vankyi L. M. Carris & L. A. Castlebury, **sp. nov.**
(Fig 1A–H)

MycoBank no.: MB 511223

Etym: Honoring Kálman Vánky, the eminent smut taxonomist.

Sori in ovario inclusi, fragiles; massa sporarum foetida, subrubeo-brunnea et pulverulenta; *cellulae steriles* globosae, subglobosae vel irregulares, 10–21 × 10.5–15 μm, hyalineae, cum parietibus 0.5–2 μm crasso, levibus; *sporae* globosae, subrubeo-brunneae, 17.5–30 × 16–22 μm, exosporium 1–2 μm crasso, reticulatum. *Basidiosporae* filiformae, 24–40 per basidium, non conjugatae, uninucleatae, 53–74 × 1.5–3.5 μm.

Typus. **Australia:** *New South Wales*: ovaries of *Lolium perenne* ssp. *perenne*, increased in greenhouse on *L. perenne* ssp. *multiflorum*, 1999, *L. M. Carris* (WSP 71263—holotypus; BPI 877335—isotypus; HUV 18929—isotypus).

Sori in ovaries of *Lolium perenne* ssp. *perenne*, enclosed by pericarp, partially hidden by palea and lemma; spore mass foetid, dark reddish brown, powdery. *Sterile cells* globose, subglobose to irregular, 10–21 × 10.5–15 μm, hyaline, wall 0.5–2 μm thick, smooth. *Teliospores* globose, medium reddish-brown, close to Umber (Rayner 1970), 17.5–30 × 16–22 μm, exospore reticulate, 1–2 μm thick, with 5–8 meshes per spore diameter. *Teliospore germination* <14 d at 5 °C and 15 °C on WA; no germination at room temperature. *Basidium* simple or branched, up to 400 μm long, with 14–34 nuclei; basidiospores 20–40 per basidium, non-conjugating, uninucleate, hyaline, filiform, 53–74 × 1.5–3.5 μm. *Sporidia* (secondary basidiospores) of two types: allantoid, 13–20 × 3–4.5 μm and filiform, 35–57 × 2–2.5 μm; both types uninucleate. Allantoid sporidia forcibly discharged, produced asymmetrically from subulate sporogenous cells, 2.5–7 × 1–2 μm, formed from hyphae, primary basidiospores and sporidia. Filiform sporidia passively released from cylindrical sporogenous cells, formed on hyphae.

Additional specimens examined in study are listed in Table 1.

Discussion

Tilletia vankyi isolates from *Festuca rubra* and *Lolium perenne* form a well-supported group in a clade with *T. laguri* and *T. lolii*. Morphologically, teliospores and sterile cells of *T. laguri* and *T. lolii* are similar to those of *T. vankyi* (Table 2; Figs 1F–H, 3A–D), differing primarily in the paler teliospore color in *T. lolii*. *T. laguri* is known from two collections, the type specimen on *Lagurus ovatus* from Italy, which was intercepted in China (Zhang *et al.* 1995), and a specimen from *L. ovatus* in Spain that was intercepted in 1982 in a shipment of dried flowers to the US (WSP 71282). We were not able to germinate teliospores of *T. laguri* from either specimen for comparison with *T. vankyi*. The germination pattern in *T. lolii* is distinct from that of *T. vankyi*, with the formation of four to 12 short, fusiform basidiospores. *T. lolii* is considered widely distributed, with records from *Lolium* spp. in Europe, Asia, and New Zealand (Zundel 1953; Lindeberg 1959; Mordue & Ainsworth 1984; Vánky 1994). However, the only published account of teliospore germination before this study was Kühn (1859), who described the teliospores as germinating in a manner similar to those of *T. caries*, but with relatively short, wide basidiospores (summarized in Liro 1938; Săvulescu 1957). Kühn's (1859) description agrees with the germination of *T. lolii* WSP 71298 (Vánky 767) as reported in this study.

We do not know whether *T. vankyi* infects the host at the seedling stage and becomes systemic, or at the floret stage, forming a localized, non-systemic infection. The bunted seeds we have examined from *Lolium* and *Festuca* were completely filled with teliospores, similar to what occurs in systemically infecting species of *Tilletia*. Most of the species of *Tilletia* studied that infect hosts in the grass subfamily *Pooideae*, including those in the present study, infect hosts systemically and replace all of the developing ovaries with teliospore-filled sori, with the notable exceptions of *T. indica* and *T. walkeri* (Castlebury *et al.* 2005; Carris *et al.* 2006). Results from the Oregon survey suggest that *T. vankyi* infects only a portion of the seeds in an inflorescence, but the illustration of infected red fescue in Hardison (1954) is more typical of a systemic bunt. Inoculations studies confirmed that *T. vankyi* is able to infect *L. perenne* ssp. *multiflorum* and ssp. *perenne* with both coleoptile and boot-stage inoculation methods. Inoculation at the coleoptile stage is used for systemically infecting species of *Tilletia*, whereas inoculation at the boot stage is generally used for nonsystemic species that infect at the floret stage. However, *T. contraversa*, a systemically infecting species, is capable of infecting susceptible hosts inoculated at the boot stage as shown in this study, so the results of artificial inoculation studies must be interpreted cautiously.

Previous studies with species of *Tilletia* have shown that a wider range of hosts are infected in inoculation studies than occur under natural conditions (Royer & Rytter 1988). Most species of *Tilletia* have a relatively narrow host range usually restricted to one genus and, in some cases, to a single host species. *T. vankyi* appears to be an exception, with hosts in two closely related genera, but additional study is needed to confirm cross-infectivity of isolates of *T. vankyi* from *F. rubra* and *L. perenne*. *Festuca* is a large, complex genus that encompasses *Lolium* spp. (Catalán *et al.* 2004). Phylogenetic analyses have shown that *L. perenne* falls within the 'broad-leaved' lineage of *Festuca*, whereas *F. rubra* is part of the 'fine-leaved' lineage (Catalán *et al.* 2004).

The earliest collection of *T. vankyi* that we have examined is on *Festuca rubra* from Oregon (WSP 71268), collected in 1951 by John R. Hardison and sent to George W. Fischer for identification. Hardison tentatively identified the fungus as the wheat dwarf bunt pathogen, *T. contraversa*, which at that time was considered a variant of *T. caries*. Subsequent publications list *F. rubra* as a host of *T. contraversa* (Hardison 1954; Durán & Fischer 1961). Hardison (1954) comments that bunted seeds were first found in seed samples of *F. rubra*, and a few infected plants were later found in a cultivated field after an

intensive search. The infected *F. rubra* plants are illustrated in Hardison (1954). The teliospores from bunted *F. rubra* seed did not germinate (Hardison 1954).

Basidiospores of *T. vankyi* have not been observed to conjugate under axenic conditions and are uninucleate based on nuclear staining using Giemsa–HCl. This would suggest that *T. vankyi* is biologically similar to *T. indica* and other species with uninucleate, non-conjugating basidiospores in which dikaryon formation and infection require proliferation of secondary basidiospores (Carris *et al.* 2006). However, unlike *T. indica*, cultures established from single basidiospores of *T. vankyi* produce masses of teliospores that are capable of germinating in a manner similar to those formed *in planta*. Infection and production of teliospores occurs in *L. perenne* and *L. perenne* ssp. *multiflorum* with inoculum derived from single basidiospores. We cannot rule out the possibility that rare conjugations occur between basidiospores, or that multinucleate basidiospores are occasionally formed, but our observations suggest that the nuclei in primary basidiospores are diploid, a condition previously reported in an inbred line of *T. caries* (Kendrick 1960), and in interspecific crosses of *Sphacelotheca* spp. (Goth *et al.* 1958). These diploid lines, called 'solopathogens' or 'solopathogenic lines', were considered to be artefacts of genetic stress induced in experimental populations (Holton *et al.* 1968). Diploids have also been reported in natural and artificially created lines of *Ustilago maydis* (reviewed in Banuett & Herskowitz 1988). Diploid lines result from the failure of the nucleus in the germinating teliospore to undergo one or both meiotic divisions, or from fusion of haploid nuclei in the basidium or primary basidiospores (Banuett & Herskowitz 1988). Additional study is underway to confirm ploidy level in basidiospores of *T. vankyi* and to determine when meiosis and karyogamy occur.

Three species that have previously been synonymized and confused with *T. contraversa* were distinguished in this study. Durán & Fischer (1961) considered *T. trabutii* a synonym of *T. contraversa*, but teliospores in collections from *Hordeum murinum* from Iran [WSP 71299 (Vánky 764)] and Australia (VPRI 32106) germinate at temperatures above 5 °C (Table 2; Ian Pascoe, pers. comm.). *Tilletia contraversa* teliospores germinate under low temperature (3–5 °C) and light in 26–60 d (Purdy *et al.* 1963). The isolates from *Hordeum murinum* form a well-supported group with *T. secalis* as the closest relative, that is distinct from the wheat bunt species in the combined analysis (Fig 4). We follow the recommendation of Ian Pascoe (pers. comm.) in using *T. trabutii* for the bunt on *H. murinum*. The isolate of *T. secalis* (WSP 71279) used in this study was derived from a volunteer plant of *Secale cereale* (cereal rye) growing in a cultivated wheat field. This collection was initially identified as *T. contraversa* based on teliospore morphology and its ability to infect wheat under greenhouse conditions (L.M.C., unpubl.). There is considerable confusion in the literature regarding *T. secalis, T. caries, T. contraversa*, and *T. laevis*, all of which are able to infect rye (Durán & Fischer 1961; Vánky 1994). The data presented in this study are the first that clearly show *T. secalis* is genetically distinct from *T. caries* and *T. contraversa*.

The two collections from *Thinopyrum intermedium* (syn *Elymus hispidus*, *Agropyron intermedium*) used in this study [HUV 20.802 and WSP 68945 (Vánky 412)] also form a distinct, well-supported species. These specimens were originally identified as *T. contraversa* based on the broad species concept proposed by Durán & Fischer (1961) and accepted by Vánky (1994). However, we do not accept this placement based on several lines of evidence. Teliospores from these two specimens germinate at temperatures up to 15 °C, and the number of basidiospores per basidium (6–8) is approximately half of those recorded for *T. contraversa* (Table 2). The teliospores (Fig 3G–H) are similar in size and ornamentation to those of *T. contraversa* (Table 2) and both taxa form teliospores with a hyaline, gelatinous sheath (Fig 3G, arrow) that extends beyond the exospore. In the *T. intermedium* bunt, most of the sterile cells also have a prominent gelatinous sheath up to 2 μm thick (Fig 3G, arrow). In contrast, sterile cells of *T. contraversa* are described as 'sometimes encased in a hyaline gelatinoid sheath 2–4 μm thick' (Durán & Fischer 1961), but this description encompasses bunts on a range of hosts including *T. intermedium*. Although teliospore and sterile cell morphology overlaps for these bunts, the germination and sequence data presented herein strongly suggest that the *T. intermedium* bunt is not conspecific with *T. contraversa*. We accept the name *Tilletia brevifaciens* for this fungus. *T. brevifaciens* was described from *Thinopyrum intermedium* (as *Agropyron intermedium*) although Fischer considered this species to also include the dwarf bunt of wheat pathogen (Fischer 1952). Conners (1954) subsequently determined the oldest valid name for the wheat dwarf bunt was *T. contraversa* and included *T. brevifaciens* as a synonym for *T. contraversa*.

The paucity of morphological features in species of *Tilletia* contributes to uncertainty in the identification and taxonomy of species in this group. Emphasis on teliospore morphology has resulted in extensive synonymy and broad host and geographic ranges for species including *T. contraversa* (Durán & Fischer 1961). In addition, molecular results suggest that single gene analyses, particularly ITS analyses, are not useful for species identifications in this group and should be interpreted with care. However, it appears that, in spite of the lack of usefulness of the ITS data alone, combining the ITS with EF1A and RPB2 improves both the resolution and support for species separation in this group (Fig 4). This study illustrates the importance of combining morphological, cultural, and multigene phylogenetic analyses in developing robust taxonomic concepts for the smut fungi.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.mycres.2007.09.008.

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